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Female meiosis is a fundamental area of study in reproductive medicine, and the mouse oocyte model of in vitro maturation (IVM) to study female meiosis is the most widely used. To investigate the probable role(s) of an unknown protein in female meiosis, the method traditionally used involves microinjecting a specific antibody into mouse oocytes. Recently, in studies on somatic cells, peptide nanoparticle-mediated antibody transfection has become a popular tool because of its high efficiency, low toxicity, good stability, and strong serum compatibility. However, till now, no researchers have tried using this technique on mouse oocytes because the zona pellucida surrounding the oocyte membrane (vitelline membrane) is usually thought or proved to be a tough barrier to macromolecules, such as antibodies and proteins. Therefore, we attempted to introduce an antibody into mouse oocytes using a peptide nanoparticle. Here we show for the first time that with our optimized method, an antibody can be effectively delivered into mouse oocytes and inhibit its target protein with high specificity. We obtained significant results using small GTPase Arl2 as a test subject protein. We propose peptide nanoparticle-mediated antibody transfection to be a superior alternative to antibody microinjection for preliminary functional studies of unknown proteins in mouse oocytes.
Effective protein inhibition in mouse oocytes through peptide nanoparticle-mediated antibody transfection

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ABSTRACT

Female meiosis is a fundamental area of study in reproductive medicine, and the mouse oocyte model of in vitro maturation (IVM) to study female meiosis is the most widely used. To investigate the probable role(s) of an unknown protein in female meiosis, the method traditionally used involves microinjecting a specific antibody into mouse oocytes. Recently, in studies on somatic cells, peptide nanoparticle-mediated antibody transfection has become a popular tool because of its high efficiency, low toxicity, good stability, and strong serum compatibility. However, till now, no researchers have tried using this technique on mouse oocytes because the zona pellucida surrounding the oocyte membrane (vitelline membrane) is usually thought or proved to be a tough barrier to macromolecules, such as antibodies and proteins. Therefore, we attempted to introduce an antibody into mouse oocytes using a peptide nanoparticle. Here we show for the first time that with our optimized method, an antibody can be effectively delivered into mouse oocytes and inhibit its target protein with high specificity. We obtained significant results using small GTPase Arl2 as a test subject protein. We propose peptide nanoparticle-mediated antibody transfection to be a superior alternative to antibody microinjection for preliminary functional studies of unknown proteins in mouse oocytes.

INTRODUCCION

Female meiosis studies in mammals are very relevant to the reproductive health of female humans and have the potential to benefit both basic reproductive medicine and clinical diagnosis.
and therapies for human reproductive disorders. However, female mammalian meiosis studies
are progressing much more slowly than general mammalian mitosis studies (publication in
PubMed, 14.1% of general). One of the biggest hurdles is that to know down a gene or inhibit a
protein, which is usually the first step of the study, researchers have to perform microinjection to
introduce siRNA or a specific antibody into oocytes. Compared with transfection, the
disadvantages of microinjection include possible mechanical damage, difficulty with dose
control, and a substantially longer time requirement.

Protein inhibition by a specific antibody is one of the most powerful tools in cell biological
studies. Compared with siRNA-mediated gene silencing, inhibition by an antibody is more
specific because the antigen–antibody association possesses the highest specificity. In addition,
protein inhibition can usually take effect much faster than siRNA silencing. Traditionally,
microinjecting the corresponding antibody into cells was the only way to inhibit a specific
subject protein (Mehlmann & Jones & Jaffe, 2002; Yin et al., 2006; Wang et al., 2008). In recent
years, antibody delivery through peptide nanoparticle-mediated transfection has emerged as a
superior alternative because of its high efficiency, low toxicity, good stability, and strong serum
compatibility (Kondo et al., 2008; Aoshiba & Yokohori & Nagai, 2003; Morris et al., 1999;
Morris et al., 2001). Another key advantage is that there are diverse commercial antibodies
available against a large portion of functionally unknown proteins in the human and mouse
proteomes (Shirai et al., 2014). However, in oocytes, whether peptide nanoparticle-mediated
antibody transfection can effectively deliver antibodies into oocytes has never been tested.

Our lab has been attempting to deliver antibodies into mouse oocytes without the use of
microinjection. After screening several peptide nanoparticle transfection reagents and testing
many protocols, we have identified the most appropriate one (Aoshiba & Yokohori & Nagai,
2003; Morris et al., 1999; Morris et al., 2001) and successfully developed a feasible standardized
approach for use with mouse oocytes.

MATERIALS AND METHODS
General chemicals & reagents and animals

Chemicals & reagents were obtained from Sigma unless otherwise stated. ICR mice used in this study were from Vitalriver experimental animal technical co., LTD of Beijing. All animal experiments were approved by the Animal Care and Use Committee of Nanjing Medical University (approval No:14030158) and were performed in accordance with institutional guidelines.

Antibodies

Rabbit polyclonal anti-Arl2 (Cat#: 10232-1-AP) and rabbit anti-Arf5 (Cat#: 20227-1-AP) were purchased from Proteintech Inc. (Chicago, IL, USA). Mouse monoclonal anti-α-tubulin antibody (Cat#:sc-8035) antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Human anti-centromere CREST antibody (Cat#: 15–234) was purchased from Antibodies Incorporated (Davis, CA, USA). Cy2-conjugated donkey anti-mouse IgG (Cat#: 715-225-150), Cy2-conjugated donkey anti-rabbit IgG (Cat#: 711-225-152) and Cy3-conjugated donkey anti-human IgG (Cat#: 711-225-152) were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA, USA).

Oocytes collection and culture

Immature oocytes arrested in prophase I (GV oocytes) were obtained from the ovaries of 3-4 week-old ICR female mice. The mice were sacrificed by cervical dislocation and ovaries were isolated and placed in operation medium (Hepes) with 2.5 nM milrinone and 10% FBS (Gibco). Oocytes were released from the ovary by puncturing the follicles with a hypodermic needle. Cumulus cells were washed off the cumulus-oocyte complexes (COC) and every 50 isolated denuded oocytes were placed in 100 µl droplets of culture medium under mineral oil (Sigma) in plastic dishes (BD). The culture medium was MEM+ (MEM with 0.01 mM EDTA, 0.23 mM Na-pyruvate, 0.2 mM pen/strep, 3 mg/ml BSA and 20% FBS). Oocytes were cultured at 37.0°C, 5% O2, 5% CO2 in humidified atmosphere. Prior to IVM (in vitro maturation), all MEM+
include 2.5 nM milrinone to prevent resumption of meiosis.

**Antibody transfection**

For antibody transfection, Chariot™ Protein Delivery Reagent (Active motif, Carlsbad, CA, USA) was used. Briefly, Two tubes, one containing 1 µl chariot (1mg / ml in 50% DMSO) in 5 µl sterile water and the other containing 1 µg antibody in PBS (final volume is also 6 µl) were first set up, then solutions from the two tubes were mixed together gently and incubated at room temperature for 30 min to allow the formation of chariot-IgG complex, then the complex solution was added into a 100 µl MEM+ drop containing 50 oocytes. After 12–14 hour treatment, the oocytes was washed to remove the complex-containing MEM+, wait for 1–2 hour and in sequence another two rounds of antibody treatment were exerted to ensure the effectiveness of the antibody inhibition. During the whole treatment, typically 40–44 hour long, 2.5 nM milrinone was always included to prevent resumption of meiosis. Next, oocytes were transfered into milrinone-free MEM+ and cultured for 8 or 16 hour, then subjected to phenotype analysis-related experiments below. Antibodies for transfection have been thoroughly buffer exchanged (over $10^4$ dilution of original buffer) into PBS / 50% glycerol to remove antiseptics (usually NaN$_3$) in the original package.

**Immunofluorescence**

Oocytes were briefly washed in PBS with 0.05% polyvinylpyrrolidone (PVP), permeated in 0.5% Triton X-100 / PHEM (60 mM PIPES, 25 mM Hepes pH 6.9, 10 mM EGTA, 8 mM MgSO$_4$) for 5 min and washed three times rapidly in PBS / PVP. Next the oocytes were fixed in 3.7% paraformaldehyde (PFA) / PHEM for 20 min, washed three times (10 min each) in PBS / PVP and blocked with blocking buffer (1% BSA / PHEM with 100 mM glycine) at room temperature for 1 hour. Then the oocytes were in sequence incubated at 4°C overnight with primary antibody diluted in blocking buffer, washed three times (10 min each) in PBS with 0.05% tween-20 (PBST), incubated at room temperature for 45 min with secondary antibody diluted in blocking
buffer (1:750 in all cases), washed three times (10 min each) in PBST. Finally DNA was stained by 10 µg / ml Hoechst 33258 and the oocytes were mounted onto a slide with mounting medium (0.5% propgal gallate, 0.1M Tris-Hcl, PH7.4, 88% Glycerol) and covered with a cover glass (0.13–0.17 µm thick). To maintain the dimension of the oocytes, two strips of double-stick tap (90 µm thick) were sticked between the slide and cover glass. Dilution of primary antibody are as follows: anti-Arl2, 1:200; anti-Arl2, 1:200; anti-α-tubulin, 1:500; anti-human centromere, 1:500. The oocytes were examined with an Andor Revolution spining disk confocal workstation (Oxford instruments, Belfast, Northern Ireland).

Data analysis and statistics

All experiments were repeated at least three times, Measurement on confocal Images was done with Image J. Data were presented as x ±Sem. Statistical comparison was done with Student’s test. P<0.05 was considered to be statistically significant.

RESULTS

A peptide nanoparticle-encapsulated antibody can effectively enter intact mouse oocytes

To test whether a peptide nanoparticle-encapsulated antibody can effectively enter intact mouse oocytes, we used a Rhodamine-conjugated control IgG and compared the cytoplasmic fluorescence of control oocytes, oocytes incubated with Rhodamine-IgG only, and oocytes incubated with peptide nanoparticle-complexed Rhodamine-IgG. We treated the GV oocytes and cultured them till MII. As shown in Figure 1A, the cytoplasmic fluorescence of control oocytes was very low, cytoplasmic fluorescence of oocytes incubated with Rhodamine-IgG only was higher than that of the controls, whereas the cytoplasmic fluorescence of oocytes incubated with peptide nanoparticle-complexed Rhodamine-IgG was significantly higher than that of the former two groups (Fig. 1A and 1B), indicating that peptide nanoparticle-complexed Rhodamine-IgG was effectively delivered into the oocytes. Furthermore, all oocytes from the three groups looked very healthy and developed to MII stage simultaneously, suggesting that the peptide nanoparticle
Peptide nanoparticle-mediated antibody transfection can specifically inhibit the target protein

To test whether a peptide nanoparticle-encapsulated antibody can specifically inhibit the target protein while at the same time not affecting others, we selected Arl2 (Arf-like 2) as a target protein and Arf5 (ADP-ribosylation factor 5) as a control protein of the same family. These proteins both belong to the GTP-binding proteins of the Ras superfamily and share high similarity (Fig. 2A). The Arl2 antibody we selected was raised against 2–182 AA of Arl2 (Fig. 2A, blue underlined), whereas the Arf5 antibody was raised against 96–106 AA of Arf5 (Fig. 2A, red underlined). Immunofluorescence showed that both proteins localize within the spindles and share similar localization patterns throughout meiosis (Fig. 2B and 2C). Thus, if Arl2 delivered via peptide nanoparticle became less specific, it could potentially bind to Arf5 as well, in which case Arf5 staining detected by a specific Arf5 antibody would significantly decrease. However, we found no significant difference between the control IgG- and anti-Arl2 antibody-treated groups (Fig.2D). This result indicates that peptide nanoparticle-mediated antibody transfection can inhibit the target protein with very high specificity, i.e., without affecting other members of the same family.

Effective protein inhibition through peptide nanoparticle-mediated antibody transfection can be used in protein function analysis

To test whether peptide nanoparticle-mediated antibody inhibition could be a powerful tool in studies of the functions of unknown proteins, we analyzed the meiotic phenotype after the antibody inhibition of Arl2. Because Arl2 mainly localizes within spindles, we hypothesized that it may function in organizing spindles so that the loss of function could affect spindle integrity and meiosis. Thus, we did a systematic phenotypic analysis on the meiotic spindles at 8 and 16 h of IVM. At 8h, there were significantly more oocytes with clumped chromosomes and without
discernable chromatids and spindle microtubules in the Arl2 inhibition group than in the control
group (Fig. 3A and 3B, control vs. Arl2, 0.95% vs. 50.18%), and we called these oocytes as
“GV-like” oocytes. There were also significantly fewer oocytes at MI (Fig. 3B, control vs. Arl2,
44.31% vs. 3.07%) in the Arl2 inhibition group than in the control group. Moreover, the spindle
length of MI oocytes in the Arl2 inhibition group was significantly shorter than that in the
control group (Fig. 3C and 3D, control vs. Arl2, 12.30 μm vs. 9.43 μm). At 16 h, there were
significantly fewer total MII oocytes (Fig. 3E and 3F, control vs. Arl2, 53.33% vs. 26.19%) in
the Arl2 inhibition group than in the control group. Furthermore, all MII oocytes in the Arl2
inhibition group also had un congressed chromosomes and kinetochores (Fig. 3E, arrow pointed),
and we called these oocytes as “Pre-MII oocytes” (Fig. 3F, control vs. Arl2, 44.15% vs. 100%).
These results indicate that Arl2 does function in organizing spindles, thereby promoting meiosis
progression.

DISCUSSION

To our knowledge, this is the first study showing that through peptide nanoparticle-mediated
antibody transfection, antibodies can effectively enter intact oocytes and inhibit specific proteins
without affecting other members of the same family. In this method, aChariot peptides form non-
covalent bonds with the antibody, stabilizes the antibody, protects it from degradation, and
preserves its natural characteristics during the transfection. After delivery, the Chariot peptide-
antibody complex dissociates and releases the antibody (Aoshi ba& Yokohori& Nagai, 2003;
Morris et al., 1999; Morris et al., 2001). We have also successfully established a standardized
protocol for the effective delivery of siRNA into mouse oocytes through peptide nanoparticle-
mediated siRNA transfection. However, the maximum reduction percentage of a target protein
by siRNA is usually only approximately 70%, and if a protein is very abundant, the remaining
protein can still be enough to retain its normal function. Therefore, the optimal approach for the
knockdown of a highly rich protein is a combination of siRNA knockdown and antibody
inhibition. If the protein content is low, antibody inhibition alone may be enough. In summary,
nanoparticle-mediated antibody inhibition is an effective approach to use when studying an
unknown protein in mouse oocytes.

Arl2 belongs to the ARF family of small GTP-binding proteins of the Ras superfamily, it
interacts with the tubulin-specific chaperone protein known as cofactor D and is involved in the
folding of tubulin peptides (Shern et al., 2003). In mitosis, Arl2 is present in centrosomes and
regulates tubulin polymerization, thereby affecting cell cycle progression (Zhou et al., 2006;
Beghin et al., 2007). However, its role in female meiosis has never been addressed. In the current
study, we found that Arl2 localized within spindles and that the inhibition of Arl2 caused
substantial spindle defects and significantly delayed meiosis progression. This demonstrates that
peptide nanoparticle-mediated antibody inhibition can be used in meiosis studies in mouse
oocytes.

In conclusion, for the first time, we have successfully developed peptide nanoparticle-
mediated antibody transfection for effective protein inhibition in mouse oocytes. We believe that
this new tool will promote further meiosis studies in mouse oocytes.

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Peptide nanoparticle-encapsulated antibody can effectively enter mouse oocytes

A, Fluorescence comparison between three different groups. Left, control oocytes without any treatment; middle, oocytes were incubated with rhodamine-conjugated rabbit IgG; right, oocytes were incubated with peptide nanoparticle-encapsulated rhodamine-conjugated rabbit IgG. B, Quantification of rhodamine fluorescence of three groups in A. Significant comparison (p < 0.05) were aster (*) marked. Scale bar, 20 µm.
Peptide nanoparticle-mediated antibody transfection can specifically inhibit target protein in mouse oocytes

A, Protein sequence alignment of Arl2 and Arf5. Blue-underlined Arl2 sequence (2–182 AA) are antigen region for anti-Arl2 antibody, red-underlined Arf5 sequence (96–106 AA) are antigen region for anti-Arf5 antibody. B, Arl2 immunolocalization in mouse oocytes at each meiotic stage. From left to right, GV, Pre-MI, MI, TI, MII. C, Arf5 immunolocalization in mouse oocytes at each meiotic stage. From left to right, GV, Pre-MI, MI, TI, MII. D, Immunostaining of Arf5 at MI stage in control IgG (left) or anti-Arl2 antibody (right) transfection group. E, Quantification of Arf5 fluorescence at MI stage in control IgG (left) or anti-Arl2 antibody (right) transfection oocytes. Scale bar, 20 µm.
Effective protein inhibition through peptide nanoparticle-mediated antibody transfection can be used in protein function analysis in mouse oocytes

A, B, At 8 hour, there were significantly more GV-like and less MI oocytes in anti-Arl2 antibody transfection group than in control. B, Percentage of oocytes at different stages at 8 hour of IVM. C, MI spindle in anti-Arl2 antibody transfection group was significantly shorter than in control. D, Quantification of MI spindle length and width in control or anti-Arl2 antibody transfection group. E and F, At 16 hour, the percentage of total MII oocytes in anti-Arl2 antibody transfection group was significantly lower than in control. And there were significantly more Pre-MI oocytes in anti-Arl2 antibody transfection group than in control. Significant comparison (p<0.05) were aster (*) marked. Scale bar, 20 µm.